Bioorganic & Medicinal Chemistry 20 (2012) 279–282

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Marine sponge *Hymeniacidon* sp. amphilectane metabolites potently inhibit rat brain microglia thromboxane B_2 generation

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ARTICLE INFO

Article history:
Received 26 August 2011
Revised 27 October 2011
Accepted 31 October 2011
Available online 9 November 2011

Keywords:
Marine
Diterpene
Microglia
Neuroinflammation
Thromboxane B_2

ABSTRACT

The effects of five *Hymeniacidon* sp. amphilectane metabolites (**1**–**5**) and two semi-synthetic analogs (**6** and **7**) on thromboxane B_2 (TXB_2) and superoxide anion (O_2^-) generation from *Escherichia coli* LPS-activated rat brain microglia were investigated. All *Hymeniacidon* sp. metabolites and analogs potently inhibited TXB_2 (IC_{50} = 0.20–4.69 μM) with low lactate dehydrogenase release and minimal mitochondrial dehydrogenase inhibition. While a lack of O_2^- inhibition would suggest that *Hymeniacidon* sp. metabolites and derivatives inhibit TXB_2 synthesis by a cyclooxygenase-dependent mechanism, their pharmacologic potency and limited in vitro cytotoxicity warrants further investigation to develop them as lead compounds to modulate enhanced TBX_2 release by activated microglia in neuroinflammatory disorders.

1. Introduction

Neuroinflammation, a complex disease process involved in the pathology of several human brain diseases, including Alzheimer's and Parkinson's disease, multiple sclerosis, ischemic stroke, HIV-dementia, trauma and infections, has been hypothesized to involve activated microglia.[1] Microglia are central nervous system mononuclear phagocytes which respond to neuropathological conditions by becoming activated and releasing large amounts of proinflammatory and potentially neurotoxic mediators, that is, proteolytic enzymes, reactive oxygen intermediates, cytokines and eicosanoids.[2] Increased release of the superoxide anion (O_2^-) and the eicosanoid thromboxane A_2 (TXA_2), an arachidonic acid metabolite which is a vasoconstrictor and platelet aggregator,[3] have been observed in activated microglia[4] as well as in neural trauma[5] and neuroinflammation.[6] Thus, the reduction or modulation of excessive O_2^- and TXB_2 generation by activated microglia has been hypothesized to contribute to the treatment and resolution of neuroinflammatory disorders.[2] Natural products isolated from marine sponges, in particular those diterpenes based on the amphilectane carbon skeleton bearing an isocyanide functionality, have been identified as potentially useful antimalarial metabolites.[7] An increasing number of such molecules have also been reported to express antimicrobial,[8] antifungal,[9] cytotoxic,[10] and antifouling activity.[11] Amphilectane-based diterpene isocyanides, on the other hand, which are typically isolated from sponges belonging to the order Halichondrida, are yet to be investigated as anti-inflammatory agents.

2. Results and discussion

The Caribbean marine sponge *Hymeniacidon* sp. was collected at Cabo Norte, Isla de Mona, at a depth of 89 feet during the summer of 2006. After the frozen sponge specimen was lyophilized, it was blended with a 1:1 mixture of $CHCl_3$–MeOH and filtered in vacuo. The crude extract was concentrated and stored under vacuum to yield an orange thick paste (28.2 g) that was partitioned with hexane (4 × 500 mL) against H_2O (1 × L). Preliminary bioassay screenings of the crude extract showed strong biological activity against *Plasmodium falciparum* (IC_{50} <0.08 μg/mL) and *Mycobacterium tuberculosis* (MIC <16 μg/mL). The hexane extract was purified by normal phase Si gel column chromatography using a gradient of increasing polarity of hexane–EtOAc (2%–50%) to afford the known diterpene isocyanides 7-isocyano-11(20)-15(16)-amphilectadiene (**1**),[12] (−)-8,15-diisocyano-11(20)-amphilectene (**2**),[13] 7,15-diisocyano-11(20)-amphilectene (**3**),[12] 8-isocyano-11(20)-ene-15-amphilectaformamide (**4**),[13] and monamphilectine A (**5**) (Fig. 1).[14]

Compounds **1**–**5** were screened for anti-neuroinflammatory activity in *Escherichia coli* lipopolysaccharide-activated rat brain microglia in vitro, specifically for thromboxane B_2 (TXB_2) and superoxide anion (O_2^-) generation inhibition. During the course of this investigation it became clear to us that isocyanide amphilectanes

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doi:10.1016/j.bmc.2011.10.086

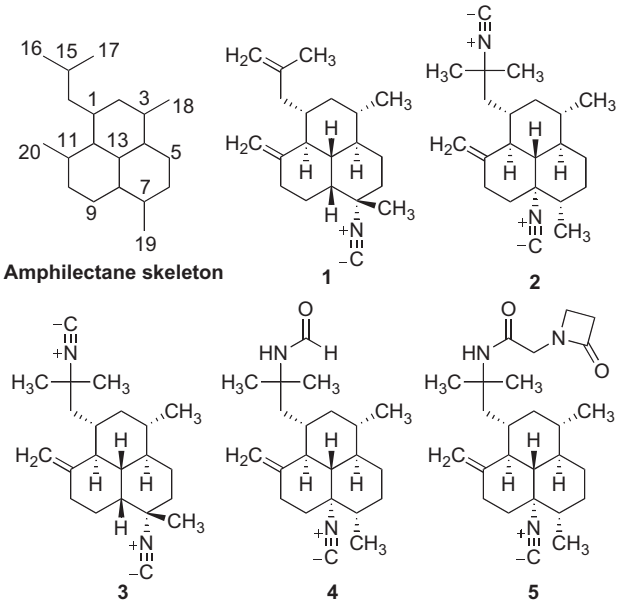
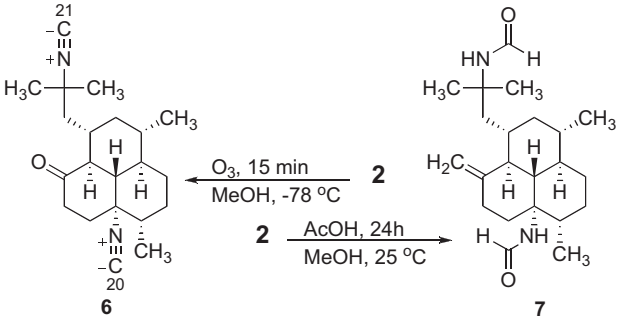


Figure 1. Amphilectane-based isocyanides isolated from the Caribbean marine sponge *Hymeniacidon* sp.

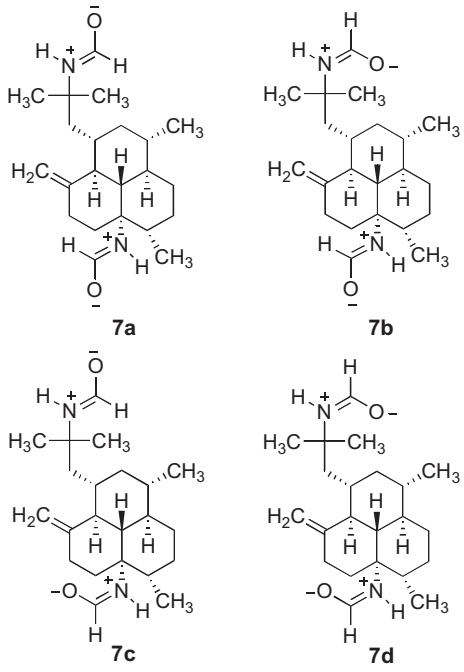
2 and **3** are in fact potent anti-neuroinflammatory agents. In order to reveal the basic structural features required for these marine diterpenes to display their biological activity, we sought to modify the molecular structure of the more abundant compound **2**.

Specifically, we wanted to scrutinize the plausible structure–activity relationships of **2** by modifying the isocyanide groups at C-8 and C-15 as well as its exocyclic alkene functionality at C-11(20). Thus, we hydrolyzed the isocyanide groups of **2** to the corresponding formamides and, in a separate experiment, oxidatively cleaved the exocyclic double bond of **2** upon ozonolysis. The structures of derivatives **6** and **7**, which were prepared using well-established procedures as shown in Scheme 1, were unambiguously demonstrated by a combination of IR, NMR (^{1}H and ^{13}C) and mass spectrometric measurements of the purified compounds. Inspection of the NMR data indicated that **7** actually consisted of an inseparable mixture of all of the four possible rotational isomers **7a–d** (Scheme 2).

Derivatives **6** and **7** along with natural products **1–5** were screened in order to determine the effect of these compounds on *E. coli* LPS-activated microglia TXB_2 and O_2^- generation in vitro. O_2^- and TXB_2 release, as well as short and long term cell viability, were assessed as described in Section 4. As shown in Table 1 and Supplementary Figure 2A, the results were as follows: all *Hymeniacidon* sp. metabolites and analogs prepared (**1–7**) potently inhibited TXB_2 generation (IC_{50} = 0.20–4.69 μM) but demonstrated minimal effect on O_2^- release (IC_{50}> 10 μM). Furthermore the compounds showed low short-term toxicity (LDH_{50} >10 μM, Supplementary Fig. 2A) and reduced long-term toxicity (WST-1 assay, Supplementary Fig. 2B). Thus, in our in vitro experimental conditions, it appeared that inhibition of microglia TXB_2 generation resulted from a pharmacologic rather than a toxic effect of compounds **1–7** on the microglia cells.



Scheme 1. Preparation of semi-synthetic analogs **6** and **7** from (−)-8-15-diisocyano-11(20)-amphilectene (**2**).



Scheme 2. Structures for the four possible rotational isomers for compound **7** detected by NMR spectroscopy.

Table 1
Anti-neuroinflammatory activity of *Hymeniacidon* sp. amphilectane metabolites[a]

Compound	O_2^- IC_{50} [μM]	TXB_2 IC_{50} [μM]	LDH_{50}[b] [μM]
1	>10	1.72	>10
2	>10	0.23	>10
3	>10	0.20	>10
4	>10	1.48	>10
5	>10	4.69	>10
6	>10	1.43	>10
7	>10	3.14	>10

[a] Effect on rat microglia PMA [1 μM]-stimulated release of O_2^-, TXB_2 and LDH. The anti-neuroinflammatory assay is described in Section 4. Data corresponds to 2–4 independent experiments.

[b] LDH_{50} represents the concentration of the compound that caused 50% release of the total LDH content of microglia cells. LDH was measured as described in Section 4.

3. Conclusion

Comparison of the IC_{50}'s of closely related amphilectane diterpenes **2** ($IC_{50} \sim 0.23$ μM) and **3** ($IC_{50} \sim 0.20$ μM) supports the observation that the observed bioactivity is associated with presence of the two isocyanide groups. However, the amphilectane diterpenoid skeleton does play a significant role, as suggested by the comparison between the IC_{50}'s of these two compounds and derivative **7** ($IC_{50} \sim 3.14$ μM), whereby each isocyanide group was replaced by a formamide functionality. Within the series of natural products **1–5**, analogs possessing an isocyanide functionality at

C-15 display the highest anti-neuroinflammatory activity, and the presence of a second isocyanide moiety within the same amphilectane core seems to further potentiate its biological activity. However, substitution at C-15 with bulkier functional groups, as observed in compound **5**, appears to lower the activity. The presence of an exocyclic alkene at C-11(20) also seems to play a role in potentiating the observed pharmacological activity. Lack of O_2^- inhibition would appear to suggest that all *Hymeniacidon* sp. metabolites and their derivatives inhibit TXB_2 synthesis through a cyclooxygenase-dependent mechanism. In addition to their pharmacologic effects on enhanced TXB_2 generation and their reduced cytotoxic effects, it is perhaps of interest to note that the *Hymeniacidon* sp. compounds are more potent inhibitors of rat microglia TXB_2 generation than acetylsalicylic acid (aspirin) (IC_{50} = 3.12–10.0 μM)[15,16] and flurbiprofen (apparent IC_{50} = 100 nM),[17] which are two clinically used NSAIDS. Thus, taken together, our present results appear to support the hypothesis that marine *Hymeniacidon* sp. metabolites and derivatives could become lead compounds for the development of novel agents to modulate excessive release of TXB_2 by activated microglia cells in neuroinflammatory disorders. This possibility remains to be investigated in in vivo pharmacologic and toxicologic investigations with these compounds.

4. Methods

4.1. General methods

Optical rotations were obtained with an Autopol IV automatic polarimeter. Infrared spectra were obtained with a Nicolet Magna FT-IR 750 spectrometer. 1D- and 2D-NMR spectra were recorded with a Bruker DRX-500 FT-NMR spectrometer. Mass spectrometric data were generated at the Mass Spectrometry Laboratory of the University of Illinois at Urbana–Champaign. Column chromatography was performed using Si gel (35–75 mesh), and TLC analysis was carried out using glass pre-coated Si gel plates and the spots were visualized using a UV lamp at λ = 254 nm or by exposure to I_2 vapor. All solvents used were either spectral grade or were distilled from glass prior to use. The identification of **1**–**5** was accomplished through rigorous comparisons with the physical and chemical data previously reported for these compounds.[12–14] The purity of each test compound was determined by NMR (^{1}H and ^{13}C) and high resolution mass spectrometric analysis or HPLC (95% >purity; see Supplementary data).

4.2. Collection, extraction, and isolation

Fresh specimens of the sponge *Hymeniacidon* sp. (Phylum Porifera; Class Demospongiae) were collected by hand using SCUBA at a depth of 89 feet off Mona Island (Cabo Norte), Puerto Rico in July 2006. A voucher specimen (No. IM06-04) is stored at the Chemistry Department of the University of Puerto Rico, Río Piedras Campus. The organism was partially air dried, frozen, and lyophilized prior to its extraction. The dry sponge (200 g) was cut into small pieces and blended using a mixture of $CHCl_3$–MeOH (1:1) (4 × 1L). After filtration, the crude extract was concentrated and stored under vacuum to yield an orange thick paste (28 g) that was suspended in H_2O (700 mL) and extracted with *n*-hexane (4 × 500 mL). The resulting hexane extract was concentrated in vacuo to yield 7.6 g of an orange oil that was purified by Si gel (170 g) column chromatography using a gradient of increasing polarity with *n*-hexane/EtOAc (98:2–1:1) as mobile phase and separated into 37 fractions on the basis of TLC and ^{1}H NMR analyses. Fraction 4 consisted of a colorless crystalline solid that was subsequently identified as known 7-isocyano-11(20)-15(16)-amphilectadiene (**1**) (10.2 mg, 0.005%).[12] Likewise, fraction 11 consisted of a colorless crystalline solid that was identified as known (−)-8,15-diisocyano-11(20)-amphilectene (**2**) (528 mg, 0.27%) after X-ray crystallographic analysis.[13] Fractions 13 and 27 were subsequently identified as known compounds 7,15-diisocyano-11(20)-amphilectene (**3**) (188.6 mg, 0.09%) and 8-isocyano-11(20)-ene-15-amphilectaformamide (**4**) (204.3 mg, 0.10%), respectively.[12,13] Fraction 36 (13.1 mg) was re-chromatographed over Si gel (1.0 g) with 20% EtOAc in *n*-hexane to afford pure monamphilectine A (**5**) (3.0 mg, 0.002%).[14]

4.3. Ozonolysis of (−)-8,15-diisocyano-11(20)-amphilectene (2)

Crystals of compound **2** (20 mg, 0.062 mmol) were dissolved in freshly distilled MeOH (20 mL) and the resulting solution was cooled to −78 °C and bubbled with O_2/O_3 for 2 min. The O_3 generator was turned off and O_2 was bubbled through the solution to remove excess ozone. The reaction was stirred for another 30 min before it was allowed to slowly warm up to 25 °C. After evaporation of the solvent, the amorphous solid left over was purified over a short column of Si gel (1.5 g) using 20% EtOAc/hexane to afford 15.6 mg (77%) of 8,15-diisocyano-11-amphilectone (**6**). Although this compound was prepared before by Ciavatta et al.,[12] the physical and chemical properties of **6** were not described.

4.3.1. 8,15-Diisocyano-11-amphilectone (6)

White solid; $[\alpha_D^{20}]$ − 20.3 (*c* 1.4, $CHCl_3$); IR (neat) ν_{max} 2968, 2925, 2870, 2127, 1714, 1460, 1444, 1371, 1166, 1149, 1116, 1087, 1060, 977, 914, 883 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.66 (1H, td, *J* = 13.5 and 6.0 Hz, H-9a), 2.42 (1H, dd, *J* = 13.7 and 5.2 Hz, H-10a), 2.36 (1H, t, *J* = 11.3 Hz, H-12), 2.24 (1H, m, H-9b), 2.19 (1H, dt, *J* = 13.5 and 3.5 Hz, H-2a), 1.95 (1H, m, H-6a), 1.90 (1H, m, H-1), 1.51 (1H, m, H-10b), 1.49 (1H, m, H-6b), 1.47 (1H, m, H-14a), 1.44 (3H, s, H-16), 1.38 (1H, m, H-5a), 1.33 (3H, s, H-17), 1.31 (1H, m, H-7), 1.27 (1H, dd, *J* = 13.8 and 9.3 Hz, H-14b), 1.19 (1H, m, H-13), 1.11 (1H, qd, *J* = 10.7 and 3.2 Hz, H-4), 0.99 (1H, m, H-3), 0.96 (3H, d, *J* = 6.1 Hz, H-19), 0.82 (3H, d, *J* = 6.4 Hz, H-18), 0.75 (1H, m, H-2b), 0.74 (1H, m, H-5b); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 210.8 (C, C-11), 157.9 (C, t, *J* = 4.3 Hz, C-20), 154.2 (C, t, *J* = 4.6 Hz, C-21), 65.7 (C, t, *J* = 4.6 Hz, C-8), 56.5 (C, t, *J* = 4.6 Hz, C-15), 55.0 (CH, C-13), 53.5 (CH, C-12), 46.7 (CH_2, C-14), 43.3 (CH, C-4), 40.4 (CH, C-7), 40.3 (CH_2, C-2), 38.5 (CH_2, C-9), 38.2 (CH_2, C-10), 35.6 (CH, C-3), 32.2 (CH_3, C-17), 31.4 (CH, C-1), 29.7 (CH_2, C-6), 29.6 (CH_2, C-5), 28.2 (CH_3, C-16), 19.6 (CH_3, C-18), 15.8 (CH_3, C-19); HRESIMS *m*/*z* $[M+Na]^+$ 349.2237 (calcd for $C_{21}H_{30}N_2ONa$, 349.2256). Recrystallization of **6** by slow evaporation from acetone gave crystals of excellent quality that were amenable to X-ray crystallographic analysis. The crystallographic data for **6** have been deposited at the Cambridge Crystallographic Data Centre, under the reference number CCDC 842072. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

4.4. Acid hydrolysis of (−)-8,15-diisocyano-11(20)-amphilectene (2)

Compound **2** (10 mg, 0.031 mmol) was dissolved in freshly distilled MeOH (10 mL), after which a few drops of glacial AcOH were added. The solution was stirred and allowed to stand at ~25 °C until all of the starting material disappeared on the basis of TLC analysis (24 h). Thereafter, the solvent was removed by vacuum distillation and the resulting solid residue was purified by flash Si gel column chromatography with 5% MeOH(NH_3)/$CHCl_3$ to afford 7.2 mg (65%) of 11(20)-ene-8,15-amphilectodiformamide (**7**). This compound consisted of an inseparable mixture of four rotational isomers about the formamide moieties, that is, **7a**–**d** (Scheme 2). Thus, a complete assignment of the ^{1}H and ^{13}C NMR signals was not feasible.

4.4.1. 11(20)-ene-8,15-Amphilectodiformamide (7)

Colorless oil; $[\alpha]_D^{20}$ – 18.0 (*c* 1.0, $CHCl_3$); IR (neat) ν_{max} 3294, 3080, 3055, 2964, 2935, 2916, 2856, 1676, 1531, 1456, 1384, 1317, 1267, 891, 754, 665 cm^{-1}; HRESIMS *m/z* $[M+Na]^+$ 383.2749 (calcd for $C_{22}H_{36}N_2O_2Na$ 383.2674).

4.5. Anti-neuroinflammatory assay

The experimental protocol to study the effect of *Hymeniacidon* sp. metabolites and semisynthetic derivatives on microglia release of O_2^- and TXB_2 was as follows. Rat neonatal microglia (2×10^5 cells) were activated in vitro by seeding the cells into each well of 24-well flat-bottom culture clusters and stimulating them with *E. coli* lipopolysaccharide (LPS) (0.3 ng/mL) for 17 h in Dulbecco's modified Eagle medium + 10% fetal bovine serum + 120 U/mL penicillin + 12 μg/mL streptomycin in a humidified 5% CO_2 incubator at 35.9 °C.[2] Media was then removed, microglia washed with warm (37 °C) Hanks' balanced salt solution (HBSS) and then microglia pre-incubated with compounds **1–7** (0.01–10 μM) or vehicle (DMSO) for 15 min prior to stimulation with phorbol 12-myristate 13-acetate (PMA) (1 μM) for 70 min. All experimental treatments were run in triplicate and in a final volume of 1 mL. After PMA stimulation HBSS was aspirated from each well and O_2^- determined via superoxide dismutase-inhibitable reduction of ferri-cytochrome C and TXB_2 by EIA as described.[2] Table 1 and Supplementary Figure 2A show data for each compound from 2 to 4 representative experiments and is expressed as the compound's inhibitory concentration 50% [IC_{50}] for O_2^- and TXB_2 generation.

4.6. Microglia cell viability assays

Short-term cell viability (90 min) was assessed by lactate dehydrogenase (LDH) release from microglia as described elsewhere.[2] Microglia LDH was expressed as percent of total LDH. Total LDH resulted from Triton X-100 (0.1%)-treated (and 100% lysed) microglia cells (intracellular LDH) plus LDH released to the extracellular medium. Table 1 and Supplementary Figure 2A show data for each compound from 3 to 4 representative experiments. *Long-term* cell viability (2.5–19 h) was determined by the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]1,3-benzene disulfonate) colorimetric assay, based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]1,3-benzene disulfonate) (slightly red) to formazan (red) by mitochondrial dehydrogenases in viable cells.[18] Briefly, rat neonatal microglia (10,000 cells/well) were plated in LPS-free 96-well cell culture clusters in 0.2 mL DMEM without FBS containing 120 U/mL P + 12 μg/mL S. Thereafter, each well received 2 μL of compounds **1–7** (0.1–10 μM final concentration) or vehicle (DMSO) and plates were incubated in a humidified 5% CO_2 incubator at 36 °C for 18 h. Thereafter, 20 μL of the tetrazolium salt WST-1 (Roche Diagnostics Indianapolis, IN) was added to wells, plates were incubated in a humidified 5% CO_2 incubator at 36 °C for 2 h, and the reduction of the WST-1 reagent to formazan was measured at 450 nM with a reference at 620 nM, with a microtiter plate (ELISA) reader. Supplementary Figure 2B illustrates data for compounds **1–7** from one representative experiment in triplicate.

4.7. Statistical analysis of the data

Data were analyzed with the Prism® software package purchased from GraphPad (San Diego, CA.). One-way analysis of variance followed by Dunnett's test was performed on all sets of data. *Hymeniacidon* sp. metabolites-treated groups were compared with the vehicle-treated group, shown as 0 or control in the corresponding figures. Differences were considered statistically significant at $p < 0.05$ and reported in each figure legend.

Acknowledgments

A.M.S.M. thanks the Office of Research and Sponsored Programs at Midwestern University for funding and Mary L. Hall for expert technical assistance. E.A. gratefully acknowledges the financial support of the GAANN and SCORE Programs of the University of Puerto Rico. We also thank Jan Vicente and the crew of the R/V Sultana for providing logistic support during the sponge collection and Dr. Raphael G. Raptis and Idaliz Rodríguez for expert assistance during X-ray crystallographic analysis of compounds **2** and **6**. Financial support from a NIH-SC1 Award to ADR (Grant 1SC1GM086271-01A1) is gratefully acknowledged.

Supplementary data

Supplementary data (copies of the NMR spectra (^1H and ^{13}C) of compounds **1–7** and Figure 2) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.086.

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